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ORIGINAL PAPER

Major physiological adjustments in freezing-tolerant grey tiger longicorn beetle (*Xylotrechus rusticus*) during overwintering period

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Abstract: [1]*Xylotrechus rusticus* (Linnaeus) is one of the most destructive woodborers in northeast China; it damages poplar and is listed as a domestic forestry quarantine pest. Overwintering larvae were collected during October 2012 and March 2013 in Harbin, China, to quantify indicators related to the insect's overwintering strategy and the major cryoprotectants. The supercooling points (SCPs), which ranged from –14.7°C to –2.9°C, were higher than the lethal temperatures of LT_{50} (–33.64°C) and LT_{99} (–40.17°C) after 24 h exposure. , also the minimum mean daily temperature (–24.5°C) and mean monthly temperature (–18.0°C) at the sampling site in January during 2008–2012. Thus, *X. rusticus* is a typical freezing-tolerant insect. Glycerol serves as a major cryoprotectant for overwintering larvae , because it was the only polyol accumulated during the winter and it also had a significant negative correlation with the SCP (p= 0.033, R =0.907). The glycogen and lipid are major sources of energy and their levels decreased substantially in the middle of overwintering, when glycogen had a significant correlation with the SCP (p= 0.006, R =0.971) whereas the lipid contents did not. Moreover, inter-conversions between glycerol and glycogen, as well as mannose and glycogen, were suggested by their negative correlations. The water content did not change obviously during the winter and was not correlated with the SCP. The free amino acids in the hemolymph and the total protein contents of the bodies of larvae changed significantly during winter, although both had no correlations with the SCP.

Keywords: cold hardiness; cryoprotectant; freezing tolerant; physiological variation; *Xylotrechus rusticus*.

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Introduction

In temperate regions, insects face a great challenge surviving in extremely low temperatures (Lee 1989; Koštál and Šimek 2000; Khani and Moharramipour 2009; Bemani et al. 2012). Their capacity for cold hardiness influences the population dynamics (Koštál et al. 2011; Woodman 2012) and geographical distribution (Payne 1926; Chen and Kang 2004; Ma et al. 2006). Therefore, studies of cold hardiness are the basis for further study of the overwintering strategies employed by insects and their cryoprotectants. In general, overwintering insects employ two major strategies, freezing-tolerant and freezing-intolerant (Lee 1989; Yohei et al. 2005; Bemani et al. 2012).

Freezing-tolerant insects recover after freezing whereas freezing-intolerant species usually fail to survive because of ice formation in their body tissues and fluids (Lee 1989; Bale 1991; Langer and Hance 2000). Bale (1996) further developed the concept of overwintering strategies and proposed that insects could be classified into five groups. In addition to the two original types, the other three groups are chill-tolerant, chill-susceptible, and opportunistic survival, which improved by considering the effects of not only freezing but also cold stress. This classification has been discussed widely in subsequent studies (Sinclair 1999; Bale 2002).

To prepare for winter, insects (freezing-tolerant and freezing-intolerant) undergo complex changes in their physiology and biochemistry (Lee 1989; Bale 2002), where low-weight molecular polyols and sugars, as well as proteins, are the most commonly induced antifreezes (Storey and Storey 1983; Li et al. 2001; Pfister and Storey 2006; Koštál et al. 2011; Kristiansen et al. 2012; Rozsypal et al. 2013). In addition, the water, lipid, and glycogen contents in the bodies of insects, as well as the free amino acid content in the hemolymph, are all known to respond

to low-temperature stress during the winter (Fields and McNeil 1988; Sinclair 2000; Li and Zachariassen 2006; Crosthwaite et al. 2011).

The grey tiger longicorn beetle, *Xylotrechus rusticus* Linnaeus (Coleoptera: Cerambycidae), is one of the most destructive pests among the woodbores and is distributed mainly in Southern Europe and Northeast Asia. This species has been found in northeast China where it is a critical threat to the safety of forests and the local populace (Li et al. 2013). The species was listed as a forestry quarantine pest by the State Forestry Administration of China after 2004. The cold hardiness of insects is an important component of studies of population dynamics but very little is known about this pest species. Thus, the present study aimed to determine the cold hardiness and major physiological variations in *X. rusticus*, thereby characterizing its overwintering strategy and major cryoprotectants.

Materials and methods

Insects

Overwintering *X. rusticus* larvae were collected from host trees (*Populus* spp.) on five occasions between October 2012 and March 2013 (October 20, 2012; November 30, 2012; January 10, 2013; February 20, 2013; and March 30, 2013) in Harbin (E 126°, N 45°), Heilongjiang Province, China. The specimens were obtained by splitting the trunks of host trees. The larvae were then transferred to the laboratory and used to determine the supercooling point (SCP), as well as to examine the physiological parameters. Larvae were also collected from the same sampling site in November 2012 and used in experiments to test their capability of exposure to low temperature.

Measurement of supercooling point (SCP)

Thirty larvae were used to determine the SCP at each of five time points during the overall overwintering period. The SCP was detected referring the method described by Atapour and Moharramipour (2009). The cooling rate was 1°C min^{-1} starting from 20°C (Ma et al. 2006; Andreadis et al. 2012).

Mortality at low temperatures

Separate larvae were exposed to eight different constant temperatures (–5, –15, –20, –25, –30, –35, –40, and –45°C) for 24 h. Each individual larva was placed in a 10-mL Eppendorf tube and kept dry. Ten individuals were used for each treatment and three replicates were performed for all treatments. After exposure to low temperatures, the specimens were maintained in standard conditions (temperature =25°C, dark: light =14:8 h, relative humidity =75%) for 24 h, before determining the absence of mandibular or body movements when stimulated with a needle (Ma et al. 2006; Atapour and Moharramipour 2009).

Mensuration of physiological indices

Six indices were measured to quantify the physiological variations in larvae at five sampling points during the overall overwintering period, i.e., the water, total lipid, glycogen, and total protein contents of the bodies of larvae, and the free amino acid, low weight molecular sugar, and polyol contents in the hemolymph.

Water and lipid contents: the fresh weight (FW) of an individual larva was measured with an electronic analytical balance (AB204-S, Mettler Toledo, Switzerland) at different stages during the overwintering period. Individual larvae were placed in 10-mL Eppendorf tubes before drying at 60°C for 72 h and they were measured again to determine the dried weight (DW). A whole larva was treated as one sample and ten larvae were used for each treatment. The water content was calculated as follows: (FW – DW) * 100/FW. The dried larva was then homogenized and its lipid contents were extracted with a chloroform-methanol (2:1) solution (Ouyang et al. 2011). The supernatant was removed after centrifugation at 3000 r/min for 10 min and this process was repeated twice. The pellet obtained was dried at 60°C for 72 h and the lean dry weight (LDW) was determined. The lipid content was calculated as follows: (DW – LDW) * 100/FW (Ouyang et al. 2011).

Total body glycogen: the FW of an individual larva was measured with an electronic analytical balance (AB204-S, Mettler Toledo, Switzerland) at five sampling time points during the overwintering period. Each individual larva was placed into a 10-mL Eppendorf tube and dried at 60°C for 72 h. The dried individual larva was homogenized with 2 mL of 70% ethanol and centrifuged at 3000 r/min for 10 min. The pooled supernatants from three replicates of this process were discarded and the remaining pellet was used to isolate glycogen, according to the method reported by Ohtsu et al. (1992). Next, 3 mL of 10% (v/v) trichloroacetic acid was added to the residue and the mixture was boiled in water for 15 min, before cooling and centrifugation at 3000 r/min for 15 min.

The supernatant was used to measure the glycogen levels. The glycogen content was determined using the phenol and sulfuric acid method (Ouyang et al. 2011). The absorbance was determined at 650 nm using a spectrophotometer (TU-1810, Pgeneral, China) and the results were expressed as mg/g (FW). A calibration curve was obtained by measuring glycogen standards at 11 concentrations ranging from 0 to 5.0 mg/mL in incremental steps of 0.5 mg/mL.

Total body protein: the FW was measured for 15 larvae with an electronic analytical balance (AB204-S, Mettler Toledo, Switzerland) at five sampling time points during the overwintering period. Individual larvae were homogenized with 5 mL phosphate-buffered saline (0.01 mol/L, pH =7.2) and centrifuged at 3000 r/min for 10 min. Each supernatant was centrifuged once more and the combined supernatant from the two replicates of this process was used to measure the total protein content. The protein content was determined according to the Bradford method (Cao and Gao 2009). The absorbance was determined at 595 nm using a spectrophotometer (TU-1810, Pgeneral, China) and

the results were expressed as μg/mL. A calibration curve was obtained by measuring protein standards at six concentrations ranging from 0 to 100 μg/mL in incremental steps of 20 μg/mL.

Free amino acid contents in hemolymph: the free amino acid content was measured by gas-liquid chromatography (Liu et al. 2007). Nine larvae were selected randomly for each treatment and 70 μL of hemolymph was sampled from each individual. The hemolymph sample was then homogenized in 1400 μL of 3% (v/v) 5-sulfosalicylic acid in a 2-mL Eppendorf tube. After centrifugation at 13,000 r/min for 15 min, the supernatant was used to measure the free amino acid levels with an amino acids auto-analyzer (L-835-50, Hitachi, Japan).

Low molecular weight sugars and polyols: 12 larvae were sampled randomly for each treatment and analyzed by gas-liquid chromatography, as described by Liu et al. (2007). Samples of 100 μL hemolymph were obtained from 4 individuals and homogenized in 400 μL of 70% (v/v) ethanol in an Eppendorf tube. After centrifugation at 10,000 r/min for 5 min, the supernatant was transferred to a 2-mL Eppendorf tube and stored at –20°C. Before analysis, the samples were dried under a stream of nitrogen at 40°C in a derivatization vial. Next, 1 mL pyridine was added to dissolve the residue, before adding 0.4 mL 6-methyl-2-silizane and 0.2 mL chlorotrimethylsilane on ice. After incubating for 6 h at 20°C, the residue was centrifuged at 10,000 r/min for 5 min, and the supernatant was used to measure the low molecular weight sugars and polyols.

Climatological data

The lowest mean daily temperature and mean monthly temperature at the sampling site in January during 2008–2012 were obtained from the China Meteorological Data Sharing Service System (http://cdc.cma.gov.cn/home.do). The ambient temperatures were used to analyze the cold tolerance and overwintering strategy of *X. rusticus*.

Statistical analysis

Differences between treatments were analyzed using ANOVA. Statistical correlations were determined using linear regression models. The mortality data from the low temperature exposure experiments were analyzed using Probit to yield Lt_{50} and Lt_{99} values. The data analyses were performed using Prism Version 6.0 (GraphPad). All of the data were expressed as the mean ± SE.

Results

Supercooling point (SCP)

The SCPs differed significantly among the five sampling time points from October 2012 to March 2013 during the overall overwintering period. The SCP tended to decrease initially as the ambient temperature declined until the middle of the overwintering period, before increasing until the end of the winter as the environmental temperature increased (Fig. 1). In all the overwintering stages, the SCPs of individuals ranged from –14.7°C to –2.9°C. The larvae had a lowest mean SCP of –11.12 ± 0.24°C at the end of November, which did not differ significantly from that of –10.82 ± 0.14°C in January, whereas relatively higher mean SCP values were detected in October (–7.94 ± 0.19°C) and in March (–5.82 ± 0.38°C).

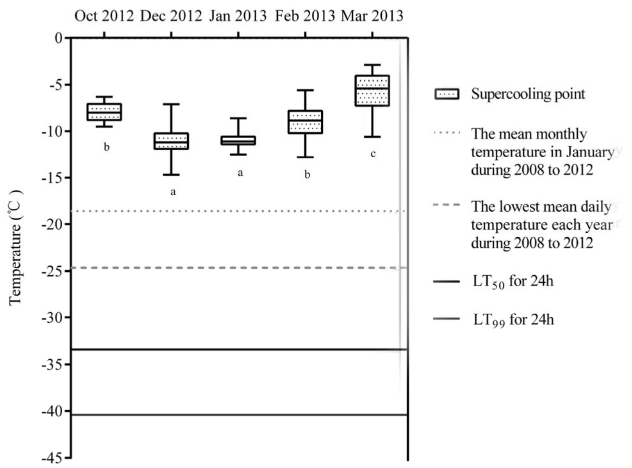

Fig. 1: Mean supercooling points of *Xylotrechus rusticus* larvae at five sampling time points during the overwintering period.

Mortality at low temperatures

The mortality of *X. rusticus* larvae increased constantly as the temperature decreased (Fig. 2). Very few deaths were caused by cold injury at temperatures above –30°C after the overwintering larvae were exposed to low temperatures for 24 h. Mortality began to occur at –30°C and increased abruptly to 76.67 ± 6.67% at –35°C. However, some overwintering larvae survived when the temperatures declined to –35°C or even lower, although none survived when the temperature decreased to –40°C, when the mortality reached 100%. Furthermore, the lethal temperatures of LT_{50} and LT_{99} were –33.64°C and –40.17°C, respectively, when the overwintering larvae were exposed to low temperatures for 24 h.

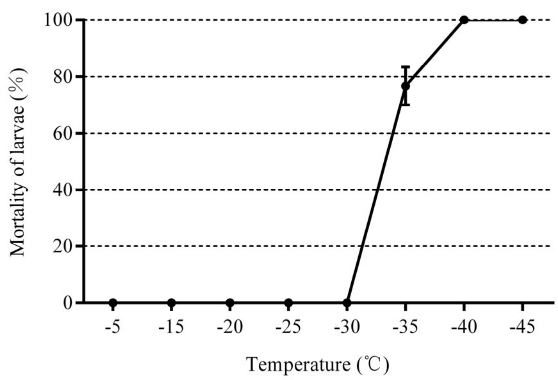

Fig. 2: Mortality of individual overwintering *Xylotrechus rusticus* larvae after exposure to eight different temperatures for 24 h.

Major physiological changes

The water content tended to increase initially, before decreasing during the winter (Fig. 3a). The mean water content of the bodies of larvae did not differ during the overall overwintering period, except when the highest level of 63.38±0.54% was reached in January. However, the maximum water content in January was only slightly higher than the minimum of 58.98±0.55% in October during the early winter.

In contrast to the water content, the total lipid content of the larval body tended to decrease at the beginning of the winter, before increasing until the end of the overwintering period (Fig. 3b). The mean lipid content differed significantly among the five sampling stages during the overall overwintering period. The lowest level of 17.17±0.27% was detected in the larvae in November, which was clearly lower than the maximum value of 22.28±0.05% in October, but only slightly lower than the mean value of 19.42±1.35% in March.

The change in the glycogen contents was similar to that in the total lipid contents, where the mean value was also significantly lower in the middle of the overwintering period (Fig. 3c). Importantly, the mean glycogen content of the larval body was almost 90% lower in the middle of the overwintering period than the mean values at the early or final stages of the overwintering period. The mean glycogen content ranged from 28.31±1.84 mg/g in March to a minimum of 2.56±0.23 mg/g in November.

The changes in the total protein content did not share the same patterns as the three indices described above, but they differed significantly between each stage of the overwintering period (Fig. 3d). The mean protein content increased from October to November, i.e., 34.93±4.59 mg/g and 66.01±2.73 mg/g, respectively, and the peak of 74.61±4.10 mg/g occurred in January. However, the total protein content then declined to a low level of 40.04 ± 1.35 mg/g in February before increasing again to a relatively high level of 61.19±2.50 mg/g in March.

Fourteen different amino acids were detected in the hemolymph of the overwintering larvae, i.e., serine (SER), glutamic acid (GLU), glycine (GLY), alanine (ALA), valine (VAL), methionine (MET), isoleucine (ILE), leucine (LEU), tyrosine (TYR), lysine (LYS), phenylalanine (PHE), histidine (HIS), arginine (ARG) and proline (PRO), but aspartate (ASP) and threonine (THR) could not be separated in the present study (Table 1). The levels of five amino acids (GLU, VAL, MET, TYR, and PRO) increased during the winter, whereas only GLU had significantly different levels between November and January, as the ambient temperature decreased.

Seven major low molecular-weight sugars and polyols were examined in the hemolymph of overwintering larvae, all of which differed significantly throughout the overall overwintering period(Table 2). The glycerol, glucose, mannose, and sorbitol contents increased clearly during November to January in the middle of the overwintering period. The glycerol content increased from a relative low level of 1835±400.0 μg/mL in October to the highest level of 6386±13.9 μg/mL in January, and then decreased to the other relatively low level of 1646±47.1 μg/mL in March. By contrast, inositol and fructose declined to their lowest levels in November. The levels of trehalose declined continuously throughout the overwintering period.

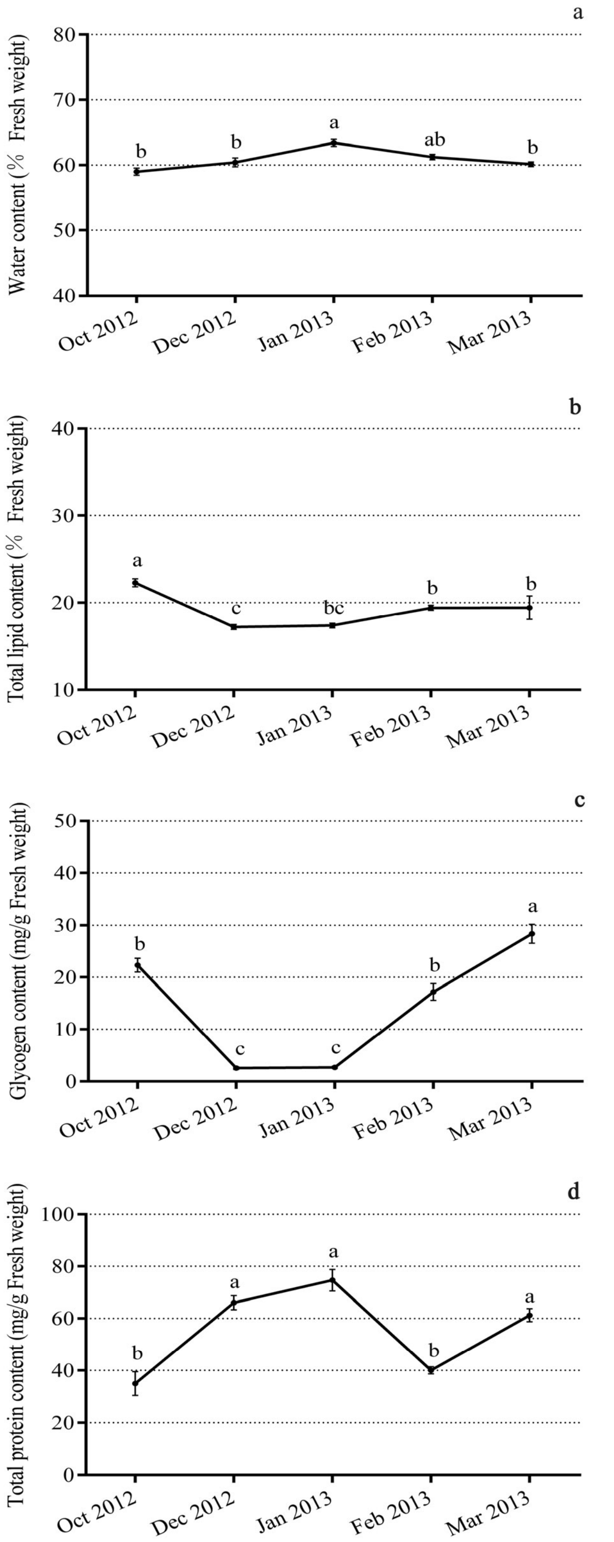

Fig. 3: Water content (a), total lipid content (b), glycogen content (c), and total protein content (d) in the bodies of overwintering *Xylotrechus rusticus* larvae at five sampling time points during the overwintering period.

Table 1: Changes in free amino acid contents in the hemolymph of *Xylotrechus rusticus* larvae at five sampling time points during the overwintering period.

Date of collection	Type of amino acid (mg/100mL; Mean±SE)							
	Aspartate and threonine	Serine	Glutamic acid	Glycine	Alanine	Yaline	Methionine	Isoleucine
Oct-12	134.21±4.17	8.93±0.67 a	0.95±0.64 a	46.30±1.77 a	196.25±5.53 a	65.61±5.74	0.00±0.00 a	39.80±4.32
Nov-12	76.24±4.87	1.53±0.11 b	37.94±2.48 b	40.74±1.57 a	107.77±14.69 b	70.63±7.86	0.43±0.11 ab	43.02±4.99
Jan-13	78.18±7.10	3.02±0.84 b	13.50±7.69 a	49.55±9.80 a	94.71±21.44 b	71.63±2.39	0.57±0.07 b	41.97±1.27
Feb-13	59.80±3.26	4.41±0.15 b	0.35±0.17 a	44.04±3.84 a	80.90±3.53 bc	77.52±5.10	2.079±0.20 c	51.19±4.36
Mar-13	89.74±0.58	19.01±1.47 c	2.41±0.25 a	7.82±1.93 b	28.81±6.53 c	64.88±4.52	0.10±0.01 ab	40.03±2.92

Date of collection	Type of amino acid (mg/100mL; Mean±SE)						
	Leucine	Tyrosine	Phenylalanine	Lysine	Histidine	Arginine	Proline
Oct-12	53.75±4.66 ab	26.68±0.61	33.52±1.12 a	92.93±3.67a	48.83±3.45 a	78.38±2.47 a	388.47±32.82 a
Nov-12	46.47±4.08 ab	26.71±2.77	21.34±2.40 b	54.50±7.06 b	39.40±3.59 b	57.79±7.29 b	420.77±36.97 a
Jan-13	50.88±1.67 ab	29.04±0.95	21.50±1.40 b	39.12±2.72 b	43.10±3.09 a	45.70±1.79 b	426.59±38.18 a
Feb-13	60.82±2.12 a	33.54±0.29	23.60±4.43 ab	59.23±11.01 b	47.19±3.38 a	46.77±4.54 b	594.94±18.04 b
Mar-13	43.59±3.52 b	30.84±2.01	21.16±0.28 b	75.91±6.62 ab	56.42±2.59 a	43.62±2.37 b	448.78±7.61 a

[§]The same letters, or data without letters, indicate no significant difference (Tukey's multiple comparisons test, $p < 0.05$).

Table 2: Changes in low molecular weight sugars and polyols in the hemolymph of *Xylotrechus rusticus* larvae at five sampling time points during the overwintering period.

Date of collection	Low weight molecular sugars and polyols (μg/mL)							
	Glycerol	Fructose	Glucose	Mannose	Sorbitol	Inositol	Trehalose	Total Content
Oct-12	1835±400.0a	181.3±11.2 a	5.9±2.0 a	0.648±0.1 a	1.454±0.5 a	122.0±8.5 a	4207±176.6 a	6353±569.9 a
Nov-12	6282±219.3b	131.4±10.2 b	17.09±1.1 b	19.72±2.7 b	6.117±0.5 ab	44.96±4.4 b	2975±114.0 b	9477±325.9 b
Jan-13	6386±13.9 b	148.2±9.0 ab	32.84±2.1 c	25.52±0.5 c	14.71±5.9 b	60.87±12.2 b	3223±46.7 b	9891±48.0 b
Feb-13	5820±84.6 b	168.5±4.6 ab	3.079±0.1 a	4.267±0.4 a	1.022±0.1 a	70.53±2.1 b	2886±62.5 b	8953±27.4 b
Mar-13	1646±47.1 a	132.1±7.2 b	1.722±0.1 a	0.638±0.04 a	0.392±0.03 a	130.0±10.2 b	2233±53.7 c	4144±83.5 c

The same letters indicate no significant difference (Tukey's multiple comparisons test, $p < 0.05$)

Discussion

Overwintering strategy

X. rusticus was suggested as a typical freezing-tolerant insect based on the SCP results and lethal temperatures of LT_{50} and LT_{95}. In the present study, the SCPs of the overwintering *X. rusticus* larvae ranged from –14.7°C to –2.9°C and the mean value was –9.2°C. Mortality due to chill injury occurred when the larvae were exposed to temperatures below –30°C for 24 h, and as low as –40°C. The LT_{50} and LT_{99} values for 24-h exposure were –33.64°C and –40.17°C, respectively. This indicated that the overwintering larvae were capable of survival after their body fluids froze at very low temperatures. Thus, the overwintering strategy of *X. rusticus* was categorized according to the classifications proposed by Bale (2002) and Lee (1989). Another freezing-tolerant insect, the beetle *Phyllodecta laticollis*, also has a high SCP of about –7°C but it can tolerate freezing to about –42°C during the winter (Laak 1982).

The overwintering strategy was also supported by the fact that *X. rusticus* larvae can survive successfully in severely cold regions during the winter. The overwintering larvae survived long-term or short-term cold stress at the sample site, where the mean monthly temperature in January during 2008–2012 was –18.0°C and the mean lowest daily temperature was –24.5°C during winter in the same 5 years. Both of these mean values were much lower than the mean SCP of –9.2°C and the minimum of –14.7°C, thereby characterizing the overwintering strategy of *X. rusticus*. These results also support the previous suggestion that a strategy of freezing-tolerance is likely to be more successful for insects in extreme winter environments (Bale 2002).

Major cryoprotectants in overwintering larvae

The results suggest that glycerol plays an important role as a major cryoprotectant in overwintering *X. rusticus* larvae because it was the only polyol accumulated in the winter and it had a significant positive correlation with SCP ($p = 0.033$, $R = 0.907$). The levels of sorbitol, mannose, and glucose were not correlated with the SCP, though they were also accumulated during the winter. *Phyllodecta laticollis* beetles (Laak 1982) and *Pytho deplanatus* larvae (Ring 1982) being freezing-tolerant insect, also increase their cold hardiness from the summer to the winter

by accumulating high concentrations of glycerol.

Glycogen and lipids were suggested as the primary energy reserves because their levels decreased greatly in overwintering larvae during late November and early January. Although glycogen contents had a strong correlation with the SCP (p =0.006, R = 0.971), the lipid contents did not. Glycogen and lipids are the two major energy reserves accumulated by most insects before overwintering (Han and Bauce 1998; Han et al. 2008; Behroozi et al. 2012). Before the onset of overwintering, *Culex tarsalis* adults synthesize large amount of lipids and approximately 78% of the total lipids comprise the main energy store for overwintering (Schaefer and Washino 1970). In addition, Behroozi et al. (2012) detected a substantial decrease in the lipid contents of overwintering *Ocneria terebinthina* larvae. Bemani et al. (2012) also suggested that *Arimania comaroffi* pupae can store energy in the form of lipids to utilize during the winter diapause.

Inter-conversions between glycerol and glycogen, as well as mannose and glycogen, were suggested by their negative correlations. Thus, there were significant relationships between the decline in the glycogen content and the accumulation of glycerol and mannose during the winter. Similarly, it has been suggested that glucose and glycogen are converted into glycerol in overwintering *Chilo suppressalis* larvae during the cold season (Atapour and Moharramipour 2009).

The lack of change in the water content agreed with a previous report that freezing-tolerant insects do not lose considerable quantities of water during cold acclimation (Ring 1982). The water contents of overwintering *X. rusticus* larvae did not change significantly, except for a slight increase in January. Moreover, there was no obvious correlation between the water content and the SCP. Li (2006) suggested that insects maintain their levels of free water during winter via an efficient water-saving mechanism.

The contributions of free amino acids to freezing-tolerance were not clear in the present study because none has significant correlations with the SCP, though the levels of GLU increased significantly during the winter. In addition, the levels of any of the free amino acids were not correlated with the protein content in the present study. Nevertheless, free amino acids play an important role in insect response to cold stress during winter. For example, overwintering larvae of *Dendrolimus spectabilis* accumulate GLU and it may be a cryoprotectant (Han et al. 2005). In addition, the level of VAL is higher in the hemolymph of cold-acclimated *Eurosta solidaginis* larvae (Storey et al. 1986), while *Sitophilus granarius* males are more cold-hardy than females because of their higher levels of PRO, ASP, and GLU (Fields et al. 1998). More detailed studies are required to elucidate the roles of free amino acids as cryoprotectants.

No significant correlations existed between the protein level and SCP in the present study, although the protein levels differed significantly during the overall overwintering period. However, most freezing-tolerant species usually produce functional proteins (ice-nucleating agents and antifreeze proteins) with apparent roles in the prevention of lethal intracellular freezing by inducing the extracellular nucleation of ice (Lee 1989; Bale 2002). Clearly, it will be necessary to investigate the metabolic pathways between proteins and free amino acids in future research.

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